STRICTOSIDINE SYNTHASE FROM CELL CULTURES OF *APOCYNACEAE* PLANTS

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1. Introduction

About 1000 monoterpenoid indole alkaloids are known to occur in higher plants which include such important members as ajmaline, quinine, strychnine, vincristine and yohimbine. All these alkaloids are formed via the condensation of tryptamine with the monoterpenoid iridoid secologanin (reviewed [1]). Recently, the key enzyme catalysing the stereospecific condensation of tryptamine with secologanin to give the glucosidal alkaloid strictosidine with 3-α (*S*) configuration was discovered [2,3]. This enzyme strictosidine synthase [3], is the starter enzyme in the pathway leading to the most diverse chemical structures found in the indole alkaloid series. The product of catalysis, strictosidine, is the universal precursor for the above-mentioned indole alkaloids [3–6].

The occurrence of strictosidine synthase in a number of cell cultures derived from *Apocynaceae* plants which are known to contain indole alkaloids is now demonstrated and some of the properties of the enzyme obtained from these origins are reported for the first time.

2. Materials and methods

2.1. *Strictosidine synthase assay*

It was observed in the course of this investigation that in the condensation reaction of tryptamine with secologanin the hydrogen atom at the C-2 position of the indole ring is lost and appears in the ambient water [7]. Therefore [*indole*-2-^{3}H]tryptamine was synthetized (2.1 μCi/μmol), and the release of tritium into the aqueous phase was used as a measure for enzyme activity. The tritium enriched water of the incubation mixture was recovered by sublimation as in [8,9]. To the sublimed frozen water 10 ml Quickszint scintillation fluid (Koch-Light) were added and the samples counted for radioactivity. Preliminary experiments using [2′-^{14}C,2-^{3}H]tryptamine as substrate for the synthase showed a 1 . 1 ratio between formation of strictosidine and HTO release. The data were therefore expressed as strictosidine formed. HTO determinations were conducted in duplicate using 50 μl incubation mixture. Standard assay conditions were as follows. 0.26 μmol (1.21 × 10^6 dpm) [2-^{3}H]tryptamine was incubated with 1.0 μmol secologanin, 20 μmol phosphate buffer (pH 7 0) and water in total vol. 0.2 ml, incubation was at 35°C. Samples were withdrawn at desirable intervals and the reaction was stopped by immediate sublimation.

2 2. *Plant cell cultures*

Plant cell cultures were provided by our cell culture laboratory. The tissue was grown in an alkaloid production medium [10] at 30°C in the dark and the cells harvested after a 7–15 day growth period. The tissue was frozen in liquid nitrogen and either used immediately or stored at −20°C.

2.3. *Enzyme preparation*

Typically frozen cells (50 g) were ground, an equal weight of Polyclar AT (wetted in the borate buffer given below) and subsequently 75 ml 0.1 M potassium borate buffer (pH 7.6) containing 20 mM mercaptoethanol, were added The mixture was stirred for 20 min. The slurry was passed through cheese-cloth and centrifuged at 48 000 × *g* for 10 min at 4°C. The supernatant was decanted and a cut was made from 35–50% saturation with solid $(NH_4)_2SO_4$. The

precipitate was collected by centrifugation and dissolved in 2 ml of 0.1 M potassium phosphate buffer (pH 7 0). To the enzyme solution was added 0.5 g Dowex 1 X 4 equilibrated in the above borate buffer, stirred for 10 min, filtered off and the supernatant treated with dextran-coated charcoal [7]. The enzyme preparation can be stored in the frozen state at −20°C for several months without loss of activity.

2.4. *Protein determination*

The amount of protein in the prepurified enzyme fractions was measured by the Folin method [11].

2.5. *Chemicals*

Secologanin was isolated by the Hutchinson procedure as in [12], [2′-^{14}C]tryptamine was from NEN, [*indole*-2-^{3}H]tryptamine was synthesized [7], and all other chemicals were obtained from commercial sources and were of the highest grade available.

3. Results

3.1. *Survey of enzyme distribution*

A number of plant cultures were grown and assayed for strictosidine synthase activity as above. Representatives of 8 alkaloid-containing genera of the family *Apocynaceae* were tested. As a control several cell cultures derived from plants which contain other alkaloids or no alkaloidal material were also investigated. The results are summarized in table 1. Enzyme activity was detected in every *Apocynaceae* species investigated and the amount and specific activity varied considerably with the species. The enzyme was completely absent from those species which did not contain indole alkaloids (only 2 species shown here) proving that this enzyme is present in and is specific for monoterpenoid indole alkaloid producing plants. Using unpurified protein fraction as an enzyme source from the above named species, in all cases the reaction product was 3-α (*S*) strictosidine. There was absolutely no indication for the formation of the 3-β (*R*) epimer vincoside.

3.2. *Properties of strictosidine synthase*

3.2.1. Rates as a function of time, protein and temperature

The release of HTO from [2-^{3}H]tryptamine which corresponds to strictosidine formation, was in all cases linear over a period of at least 30 min. In some cases (e.g., *Amsonia*) a distinct lag was observed before the rate of reaction became constant. The HTO release continued until the limiting substrate was completely utilized. The rate of reaction was linear with 50–300 μg protein. The temperature optimum for the reaction was found to be between 45–50°C for the synthase of all species listed in table 1.

Table 1
Survey of distribution of strictosidine synthase activity in species of different genera of the family *Apocynaceae* and two unrelated species

Plant material	Family	Enzyme activity pkat/g cells (dry wt)	pkat/mg protein
Amsonia salicifolia	Apocynaceae	912	210
Catharanthus roseus	Apocynaceae	1801	340
Ochrosia elliptica	Apocynaceae	532	1698
Rauwolfia vomitoria	Apocynaceae	5089	773
Rhazia orientalis	Apocynaceae	1785	357
Stemmadenia tomentosa	Apocynaceae	555	166
Vinca minor	Apocynaceae	556	146
Voacanga africana	Apocynaceae	3386	1229
Nicotiana tabacum	Solanaceae	0	0
Trifolium pratense	Leguminosae	0	0

The assay method and reaction mixture were as in section 2. The assay time was 45 min and the 35–50% $(NH_4)_2SO_4$ protein fraction was used as enzyme source

3.2.2. pH Optimum

As noted in [2,3], the enzyme shows activity even at a pH value as low as 4.1. Using the assay presented here a complete pH curve was established for the synthase reaction. Using a 35–50% $(NH_4)_2SO_4$ fraction from *Amsonia salicifolia* cells as enzyme source, a broad pH profile was found with optimum activity between pH 6.5 and 7.0, with phosphate as the buffer as shown in fig.1. The buffer used had a considerable effect. At pH 7.0, the activity found with Tris–HCl was 0.7-times that found with a phosphate buffer.

3.2.3. Effect of substrate concentration

While the enzyme exhibited normal Michaelis-Menten kinetics with secologanin as substrate, there was marked substrate inhibition by tryptamine at >1 mM (fig.2). The K_m values were calculated using a computer program for linear regression analysis of a straight line form of the Michaelis-Menten equation. They were found to be 2.6 mM for secologanin and 5.8 mM for tryptamine for a preparation of the enzyme from *Rauwolfia vomitoria* (fig.2).

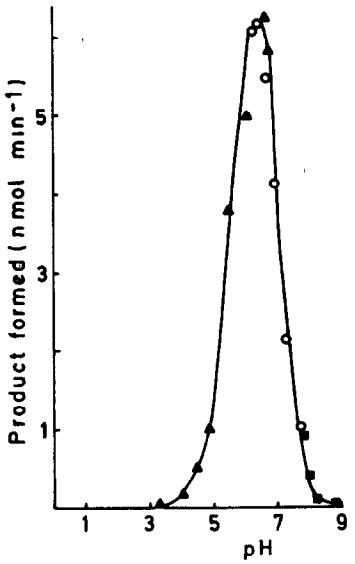

Fig 1. pH Profile of the catalytic activity strictosidine synthase from *Amsonia salicifolia* cell cultures. The assay was conducted in the presence of 0.1 M citrate/phosphate (▲), KPO_4^{2-} (○) and borate (■) buffer, respectively.

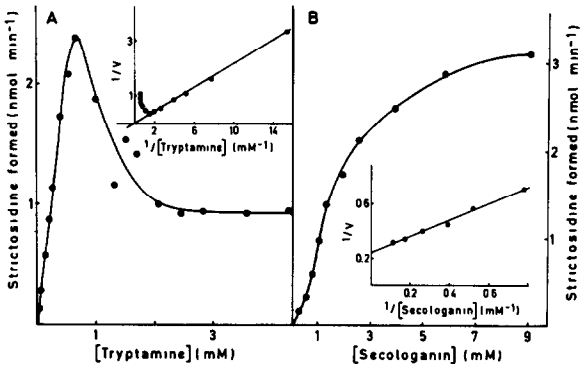

Fig.2 Reaction rate of strictosidine synthase from *Rauwolfia vomitoria* as a function of tryptamine (A) and secologanin (B) concentration. The reaction conditions were as in section 2 except that the reaction time was 45 min. The standard reaction mixture (0 2 ml final vol.) was used, except that the amount of tryptamine (A) or secologanin (B) was varied as noted. Of the 35–50% $(NH_4)_2SO_4$ fraction, 55 μg (850 pkat/mg) protein was used in (A) and 82 μg (720 pkat/mg) in (B).

3.2.4. Enzyme inhibitor studies

The enzyme was not inhibited by the following potential inhibitors at final 10^{-2} M: iodosobenzoate; 5,5′-dithiobis-(2-nitrobenzoic acid), δ-gluconolactone; *N*-ethylmaleimide, iodoacetamide; Zn^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+}; in addition no inhibition of the reaction was found by L- or D-tryptophan. Only *p*-chloromercurybenzoate was found to inhibit the enzyme ~70% at 10^{-3} M. Compounds which react with the carbonyl function of secologanin inhibit the reaction by inactivating the substrate.

3.2.5. Molecular weight determinations

Using calibrated Sephadex G-100 columns and bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome *c* as marker proteins the molecular weights of strictosidin synthases from 7 different plant species were found to be without any exception from mol. wt 26 000–33 000. This low molecular weight suggests that the enzyme may consist of only one catalytic entity and subunits may be missing.

4. Conclusion

The novel enzyme strictosidine synthase has now been proven to be of common occurrence in monoter-

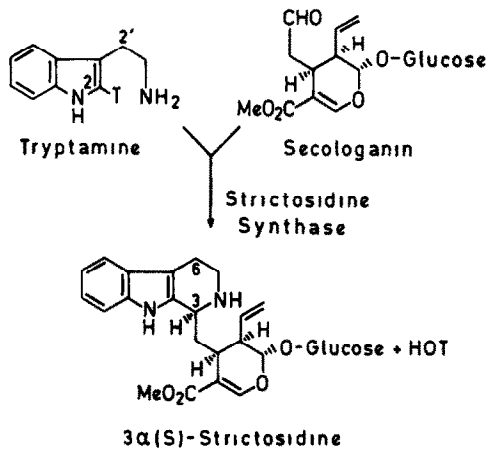

Fig.3. Reaction sequence catalysed by strictosidine synthase indicating also the assay principle.

penoid indole alkaloid containing *Apocynaceae* plants. The enzyme catalyses the stereospecific condensation of tryptamine with secologanin with the formation of exclusively 3-α (*S*) strictosidine [2,3] as shown in fig.3. By this Pictet-Spengler-type reaction a tetrahydro-β-carboline is formed under elimination of the hydrogen atom at C-2 of the indole ring. Using [*indole*-2-^{3}H]tryptamine the enzymatically-catalysed elimination of tritium makes a convenient and sensitive quantitative test for this enzyme. The enzyme has a low molecular weight, ~30 000, and does not seem to be a site of substrate or product regulation, it is, however, inhibited by excessive concentrations of tryptamine The pH optimum of the synthase of 8 species belonging to different genera of the *Apocynaceae* is between pH 6.0 and 7.0. This is in accordance with our report on the overall reaction leading from tryptamine and secologanin via strictosidine to ajmalicine and its isomers 19-epi-ajmalicine and tetrahydroalstonine, which also shows a pH optimum at 6.5 [13]. The crude and partially purified enzyme is remarkably stable and shows a temperature optimum at 45°C. Strictosidine synthase occupies a central position in the formation of the multitude of monoterpenoid indole alkaloids some of which have high commercial interest. Because of the interest especially in the *Catharanthus* alkaloids [1] the purification and characterization of the synthase from this plant source will be detailed in [7].

Acknowledgements

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